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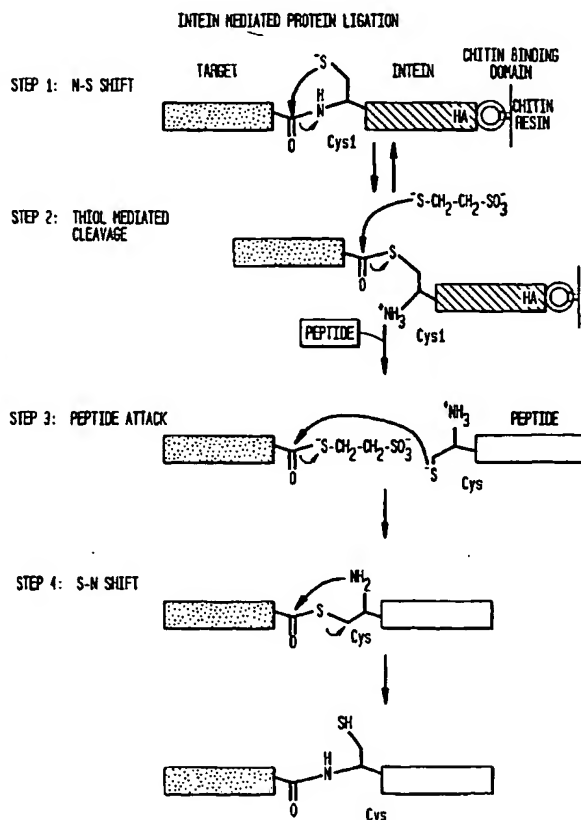
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(54) Title: INTEIN MEDIATED PEPTIDE LIGATION



(57) Abstract: An in vitro method for producing a semi-synthetic fusion protein is provided, whereby a target protein fused to an intein - a protein splicing element - is selectively cleaved in a first step as depicted in Figure 1 with a thiol reagent, forming a carboxyl-terminal thioester of the target protein and releasing the target protein from the intein. In a subsequent step as shown in Figure 1, a desired, synthetic, protein or peptide having an amino-terminal cysteine is ligated to the target protein. Standard thiol-reagents such as DTT, or thiol-reagents optimized for ligation such as the odorless MESNA, may be used in the first step. The method permits the direct ligation of a desired peptide to a thioester bond that had linked a target protein to an intein. An in vivo variation of the method will permit production of a cytotoxic protein: a truncated, inactive, form of the protein fused to an intein is introduced in vivo, this fusion product is then selectively cleaved, and a synthetic protein or peptide is subsequently ligated at a carboxyl-terminal thioester of the target protein in order to restore the native activity of the cytotoxic protein.

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## **INTEIN MEDIATED PEPTIDE LIGATION**

### **BACKGROUND OF THE INVENTION**

5           Genetic engineering is a powerful approach to the manipulation of proteins. However, genetic methodologies are constrained by the use of only naturally coded amino acids. Furthermore, cytotoxic proteins are difficult to obtain by expression and isolation from a living source, since the  
10           expression of the toxic protein can result in death of the host.

          To some extent, protocols have been developed to circumvent these problems, for example, total chemical synthesis (Kent, S. B. (1988) *Ann. Rev. Biochem.* 57:957-989),  
15           use of misacylated tRNAs (Noren, et al., (1989) *Science* 244:182-188), and semi-synthetic techniques (reviewed in Offord, R. (1987) *Protein Eng.* 1:151-157; Roy. et al. (1994) *Methods in Enzymol.* 231:194-215; Wallace, C. J. (1993) *FASEB* 7:505-515). However, all of these procedures are limited by  
20           either the size of the fragment which can be generated or by low reaction yield.

          It would therefore be desirable to develop a high-yield, semi-synthetic technique to allow *in vitro* fusion of a  
25           synthetic protein or peptide fragment to an expressed protein without limitation as to the size of the fused fragments.

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Likewise, in order to produce cytotoxic proteins, it would be desirable to develop a method of fusing a synthetic fragment, *in vitro*, to an inactive, expressed protein, so as to restore protein activity post-production from the host.

The modified Sce VMA intein has been used to generate thioester-tagged proteins for use in ligation (Example 19, U.S.S.N. 08/811,492, filed June 16, 1997; Chong, (1996) *J. Biol. Chem.*, 271(36):22159-22168; Chong, (1997) *Gene*, 192:271-281; and Muir, et al. (1998) *Proc. Natl. Acad. Sci USA* 95:6705-6710).

Some disadvantages have been low yields due to poor cleavage of the Sce VMA intein with thiol-reagents that are optimum for ligation, the need for large peptide quantities due to on-column reactions, the use of odoriferous reagents, and/or low protein yields due to the use of a large, eukaryotic intein.

### SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method for producing a semi-synthetic fusion protein *in vitro*, comprising the steps of producing a target protein fused to a protein splicing element (an intein) and selectively cleaving the fusion and ligating a synthetic

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protein or peptide at the C-terminal thioester of the target protein, which overcome many of the disadvantages and problems noted above. Specifically, the present invention has higher yields due to better thiol-induced cleavage with thiol reagents which have been optimized for the ligation reaction. Off-column ligation allows for sample concentration as well as the use of less peptide. In a particularly preferred embodiment, thiol reagents such as 2-mercaptoethanesulfonic acid (MESNA), which is an odorless thiol-reagent, is used for cleavage and ligation along with the Mxe intein, which is from a bacterial source and often expresses better in bacterial cells. Furthermore, the present invention allows peptides to be directly ligated to the thioester bond formed between an intein and the target protein. The present invention also provides a method for producing a cytotoxic protein, comprising the steps of producing a truncated, inactive form of the protein *in vivo* which is fused to a protein splicing element, and selectively cleaving the fusion and ligating a synthetic protein or peptide at a C-terminal thioester of the target protein to restore the activity of the native cytotoxic protein. Recombinant vectors for producing such cleavable fusion proteins are also provided.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a flow diagram depicting the chemical reactions which enable intein-mediated peptide ligation. The

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thioester generated at the C-terminus of the target protein during IMPACT™ purification was used in a 'native chemical ligation' reaction. This allowed the ligation of a synthetic peptide to a bacterially expressed protein. A typical ligation reaction involved the expression of the target protein-intein-CBD fusion followed by binding to a chitin resin. A thiol reagent induced cleavage of the intein. The target was eluted from the chitin resin and a synthetic peptide was added. The ligation reaction proceeded overnight.

Figure 2 is a gel depicting the results of cleavage and ligation reactions using various thiols. Cleavage and ligation reactions with different thiols visualized on 10-20% Tricine gels. MYB (a fusion protein of maltose binding protein-Sce VMA intein (N454A)-chitin binding domain) and MXB (a fusion protein of maltose binding protein-Mxe GyrA (N198A) intein-chitin binding domain) were incubated overnight at 4°C with various thiols (50 mM) in 150 mM Tris, 100 mM NaCl, pH 8 in the presence of a 30 amino acid peptide with an N-terminal cysteine. The peptide ligates to the C-terminus of MBP. Lanes 1-5 ligation with MYB. Lane 1 no thiol. Lane 2 dithiothreitol. Lane 3 2-mercaptoethanesulfonic acid. Lane 4 3-mercaptopropionic acid. Lane 5 thiophenol. Lanes 6-10 ligation with MXB. Lane 6 no thiol. Lane 7 dithiothreitol. Lane 8 2-mercaptoethanesulfonic acid. Lane 9 3-mercaptopropionic acid. Lane 10 thiophenol.

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Figure 3 is a gel depicting direct ligation of a peptide to the thioester formed between the Sce VMA intein and maltose binding protein. SDS-PAGE of direct ligation reaction with a 10-20% Tricine gel. Lane 1: a precursor protein (MYBleu) consisting of maltose binding protein-Sce VMA1 intein-chitin binding domain was heated to >95°C for 5 minutes in a buffer of 50 mM Trizma base, pH 8.5 containing 100 mM NaCl, 1% SDS, and mM tris-(2-carboxyethyl)phosphine (TCEP) followed by overnight incubation at room temperature. The precursor (MYBleu) is visible along with the Sce VMA1 intein (Y) and maltose binding protein (M), which are cleavage products. Lane 2: the precursor protein was subjected to the same conditions as described in Lane 1 except that the 30 amino acid peptide (1 mM) was added. The precursor (MYB) and cleavage products (Y and M) are visible along with the ligation product (M+30mer) formed when the 30 amino acid peptide fuses to maltose binding protein.

Figure 4 is a diagram depicting the pTXB1 expression vector of Example I (SEQ ID NO:7 and SEQ ID NO:8).

Figure 5 is the DNA sequence of pTXB1 (SEQ ID NO:5).

Figure 6 is a gel depicting the results of the *HpaI* protein ligation reaction. Protein ligation reactions examined on 10-20% Tricine gels. Lane 1: clarified cells extract after IPTG (0.5 mM) induction of ER2566 cells containing the pTXB2-*HpaI*

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plasmid. The fusion protein of *HpaI*<sub>223</sub>-Mxe GyrA-intein-CBD (52 kDa) is visible. Lane 2: cell extract as in Lane 1 after passage over a chitin column, which results in the binding of the fusion protein. Lane 3: *HpaI*<sub>223</sub> (25.7 kDa) after cleavage from the fusion protein by addition of MESNA. Lane 4: ligation product of *HpaI*<sub>223</sub> (0.2 mg/mL) with 1 mM of a 31 amino acid peptide (ligation product 29.6 kDa), representing the residues necessary to generate full length *HpaI*, after overnight incubation at 4°C. Lane 5: full length *HpaI* from a recombinant source (29.6 kDa) containing BSA (66 kDa) and two impurities.

Figure 7 is a western blot of various proteins ligated to a biotinylated peptide. Proteins purified with the Mxe GyrA IMPACT™ derivative were ligated to a synthetic peptide which contained an antibody recognition sequence.

### DETAILED DESCRIPTION OF THE INVENTION

The ligation methods of the present invention are based on the discovery that a cysteine or peptide fragment containing an N-terminal cysteine may be fused, *in vitro*, to a bacterially expressed protein produced by thiol-induced cleavage of an intein (U.S. Patent No. 5,496,714; Example 19 of U.S.S.N. 08/811,492 filed June 16, 1997; Chong, et al., (1996) *supra* and Chong, et al., (1997) *supra*.



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The ligation procedure disclosed herein utilizes a protein splicing element, an intein (Perler, et al., (1994) *Nucleic Acids Res.* 22:1125-1127) to precisely create a thioester at the C-terminal  $\alpha$ -carbon of an expressed protein.

5 This reactive thioester could be present between the target protein and intein or generated by the addition of a thiol reagent. Previously the generation such a thioester was described using an intein (CIVPS) that was modified to undergo thiol inducible cleavage at its N-terminal junction in  
10 the presence of thiol reagent dithiothreitol (DTT) (Chong, et al. (1997) *supra*; Comb, et.al. U.S. Patent No. 5,496,714). This C-terminal thioester was previously used in a 'native chemical ligation' type reaction (Dawson, et al., (1994) *Science* 266:776-779) to fuse  $^{35}\text{S}$ -cysteine or a peptide  
15 fragment containing an N-terminal cysteine to a bacterially expressed protein (Example 19, Comb, et.al. U.S. Patent No. 5,834,247, Chong (1996) *supra* and Chong (1997) *supra*.

20 The ligation method of the instant invention begins with the purification of the thioester-tagged target protein using an intein as described (Chong, et.al. (1997) *supra*). The direct ligation method of the instant invention begins with the isolation of a precursor composed of the target protein-intein-CBD. In one preferred embodiment, the host cell is  
25 bacterial. In other embodiments the host cell may be yeast, insect, or mammalian. A cysteine thiol at the N-terminus of a synthetic peptide nucleophilically attacks a thioester present

on the freshly isolated C-terminal  $\alpha$ -carbon of the target protein or directly attacks the thioester present between the target protein and intein. This initially generates a thioester between the two reactants which spontaneously rearranges into a native peptide bond (Figure 1).

In order to optimize the ligation efficiency so that greater than 90% of the bacterially expressed target protein can be fused to the synthetic peptide or protein, specific thiol reagents and inteins are screened. In a preferred embodiment, the intein may be any CIVPS, such as *Sce* VMA, *Mxe* GyrA or derivatives of mutants thereof, and the thiol reagent is 2-mercapto-ethanesulfonic acid, thiophenol, DTT, or 3-mercaptopropionic acid (Comb, et al., U.S. Patent No. 5,496,714; U.S. Patent No. 5,834,247).

In one particularly preferred embodiment, an intein whose protein splicing activity has been blocked by mutation is utilized. The mutant must, however, retain the ability to undergo the N-S shift, thus allowing thioester formation between itself and an N-terminal protein. This thioester can then be nucleophilically attacked by a thiol reagent or by the N-terminal cysteine of a peptide sequence. For example, by mutating the C-terminal asparagine (asn 198) of an intein from the GyrA gene of *Mycobacterium xenopi* (Telenti, et al., (1997) *J Bacteriol* 179:6378-6382) to an alanine created a

thiol inducible cleavage element. This modified intein cleaved well with thiol reagents that were optimal for the ligation reaction, such as MESNA and thiophenol. Furthermore, optimal thiol reagent and intein combinations can be determined by incubating a precursor protein containing the intein of interest with a wide variety of thiol reagents followed by determination of the extent of cleavage of the precursor protein (Figure 2).

The use of such intein and specific thiol reagents leads to optimal yields and high ligation efficiencies; typically greater than 90% of the N-terminal ligation fragment can be modified.

The ligation methods of the present invention expand the ability to incorporate non-coded amino acids into large protein sequences by generating a synthetic peptide fragment with fluorescent probes, spin labels, affinity tags, radiolabels, or antigenic determinants and ligating this to an *in vivo* expressed protein isolated using a modified intein.

Furthermore, this procedure allows the isolation of cytotoxic proteins by purifying an inactive truncated precursor from a host source, for example bacteria, and generating an active protein or enzyme after the ligation of a synthetic peptide. For example, restriction endonucleases which have not successfully been cloned by traditional

methods may be produced in accordance with the present invention.

Also, the direct ligation procedure allows the ligation of  
5 a protein or peptide sequence to another protein or peptide  
sequence without the use of exogenous thiol reagents. Direct  
ligation relies on the nucleophilic attack of the N-terminal  
amino acid of one peptide on the thioester formed between a  
target protein and an intein (Figure 3).

10 In summary, a fusion protein can be created using the  
methods of the present invention that possesses unique  
properties which, currently, can not be generated genetically.

15 The Examples presented below are only intended as  
specific preferred embodiments of the present invention and  
are not intended to limit the scope of the invention. The  
present invention encompasses modifications and variations  
of the methods taught herein which would be obvious to one of  
20 ordinary skill in the art.

The references cited above and below are herein  
incorporated by reference.

**EXAMPLE I****Creation of vectors pTXB1 and pTXB2 for ligation:**

5           Asparagine 198 of the *Mxe* GyrA intein (Telenti, et al., (1997) *J Bacteriol.* 179:6378-6382) was mutated to alanine by linker insertion into the *XmnI* and *PstI* sites of pmxeMIPTyrXmnSPdel to create pMXP1. The *XmnI* site was originally introduced into the unmodified *Mxe* GyrA intein  
10           sequence by silent mutagenesis. The *PstI* site was a unique site in the plasmid. The linker was composed of mxe#3 (5'-GGTTCGTCAGCCACGCTACTGGCCTCACCGGTTGATAGCTGCA-3') (SEQ ID NO:1) and mxe#4 (5'-GCTATCAACCGGTGAGGCCAGTAGCGTGGCTGACGAACC-3') (SEQ ID NO:2).

15           Into pMXP1 another linker composed of mxe#1 (5'-TCGAATCTAGACATATGGCCATGGGTGGCGGCCGCTCGAGGGCTCTTCTGCATCACGGGAGATGCA-3') (SEQ ID NO:3) and mxe#2 (5'-CTAGTGCATCTCCCGTGATGCAGGAAGAGCCCTCGAGGCGHGCGCCACCCA  
20           TGGCCATATGTCTAGAT-3') (SEQ ID NO:4) was inserted into the *XhoI* and *SpeI* sites to introduce a multiple cloning site (*XbaI*-*NdeI*-*NcoI*-*NotI*-*XhoI*-*SapI*) before the *Mxe* GyrA intein (pMXP2).

25           The 0.6 kilobase *NotI* to *AgeI* fragment of pMXP2 was ligated into the same sites in pTYB1 (IMPACT kit, New England Biolabs, Beverly, MA) and the *NcoI* to *AgeI* fragment of pMXP2

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was cloned into pTYB3 (IMPACT kit, New England Biolabs, Beverly, MA) to create plasmids pTXB1 (see Figure 4 and 5) (SEQ ID NO:5) and pTXB2, respectively. These vectors have a multiple cloning site upstream of the modified *Mxe* GyrA intein-chitin binding domain fusion. This allows the insertion of a target gene of interest inframe with the intein and chitin binding domain (CBD).

#### **Creation of vectors pMYBleu for ligation:**

pMYBleu was as described in Chong, et al., (1998), *J. Biol. Chem.* 273:10567-10577. This vector consisted of maltose binding protein upstream of the *Sce* VMA intein-chitin binding domain. A leucine is present at the -1 position instead of the native residue (which is a glycine).

#### **Purification of Thioester-Tagged Proteins:**

Protein purification was as described using the *Sce* VMA intein (Chong, et.al., (1997) *Gene* 192:271-281) with slight modification. ER2566 cells (IMPACT T7 instruction manual from New England Biolabs, Beverly, MA) containing the pTXB vector with the appropriate insert were grown to an OD<sub>600</sub> of 0.5-0.6 at 37°C at which point they were induced with 0.5 mM IPTG overnight at 15°C. Cells were harvested by centrifugation and lysed by sonication (performed on ice). The

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three part fusion protein was bound to chitin beads (10 mL bed volume, Figure 6, lanes 1 and 2) equilibrated in Buffer A (50 mM Tris, pH 7.4, and 500 mM NaCl), and washed with 10 column volumes of Buffer A to remove unbound material.

5

Cleavage was initiated using a buffer of 50 mM 2-mercaptoethanesulfonic acid (MESNA), 50 mM Tris, pH 8.0 and 100 mM NaCl. Other thiol reagents were also used at other times, such as thiophenol, dithiothreitol, and/or 3-mercaptopropionic acid. After overnight incubation at from 4-25°C protein was eluted from the column (Figure 6 lane 3). This protein contained a thioester at the C-terminus.

10

#### **Purification of MYB, MYBleu and MXB:**

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Full length precursor proteins consisting of maltose binding protein-*Sce* VMA intein (N454A)-chitin binding domain (MYB) and maltose binding protein-*Mxe* GyrA (N198A) intein-chitin binding domain (MXB) were purified after induction and sonication, as described above, by applying the sonicated sample to a 10 mL column of amylose resin (New England Biolabs, Beverly, MA). Unbound proteins were washed from the column with 10 column volumes of Buffer A (see purification of thioester-tagged proteins). Bound proteins were eluted with a buffer of 50 mM Tris, pH 8, containing 100 mM NaCl and 10 mM maltose. Fractions were collected and protein

20

25

concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA).

### Peptide Synthesis:

5

Peptides for subsequent ligation reactions were synthesized on an ABI model 433A peptide synthesizer utilizing *FastMoc*<sup>™</sup> chemistry (Fields, et al., (1991) *Pept Res* 4, 95-101) at a 0.085 mmol scale. Preloaded HMP (p-  
10 hydroxymethylphenoxymethyl) polystyrene resins (Applied Biosystems, Foster City, CA) functionalized at 0.5 mmol/g was used in conjunction with Fmoc/NMP chemistry utilizing HBTU amino acid activation (Dourtoglou, et al., (1984) *Synthesis* 572-574; Knorr, et al., (1989) *Tetrahedron Lett* 30,  
15 1927-1930). Fmoc amino acids were purchased from Applied Biosystems (Foster City, CA).

Synthesis proceeded with a single coupling during each cycle. Peptide cleavage from the resin and simultaneous  
20 removal of side chain protecting groups was facilitated by the addition of cleavage mixture (Perkin Elmer, Norwalk, CT) consisting of 0.75 g phenol, 0.25 mL 1,2-ethanedithiol, 0.5 mL deionized H<sub>2</sub>O, and 10 mL TFA. The resin was flushed with nitrogen and gently stirred at room temperature for 3 hours.  
25 Following filtration and precipitation into cold (0°C) methyl-t-butyl ether, the precipitate in the ether fraction was



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collected by centrifugation. The peptide precipitate was vacuum dried and analyzed by mass spectrometry using a Perceptive Biosystems (Framingham, MA) MALDI-TOF mass spectrometer.

5

Final purification was by HPLC using a Waters HPLC system with a Lambda-Max Model 481 Multiwavelength detector (set at 214 nm), 500 series pumps and automated gradient controller with a Vydac semi-preparative C18 column. Elution of the peptide was with a 60 minute linear gradient of 6-60% acetonitrile (v/v) in an aqueous solution of 0.1% TFA (v/v).

10

#### **Protein Cleavage and Ligation Reactions:**

15

Cleavage of MYB and MXB: The precursor protein (1 mg/mL) was incubated overnight at 4°C with or without a thiol reagent (50 mM) in 150 mM Tris, pH 8, containing 100 mM NaCl.

20

Ligation reactions with MYB and MXB: The precursor protein (1 mg/mL) was treated as described for cleavage except that a 30 amino acid peptide (1 mM final concentration, NH<sub>2</sub>-CAYKTTQANKHIIIVACEGNPYVPVHFDAV-COOH (SEQ ID NO:6) was also included in the reaction (Figure 2).

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Ligation reactions after purification of thioester-tagged proteins: Lyophilized peptides (New England Biolabs, Beverly, MA) were added (to 1 mM final concentration) directly to the thioester-tagged protein freshly isolated from the chitin column. The reaction was allowed to proceed overnight at from 4-25°C. In both ligation procedures the condensation of the reactants is visible on a 10-20% Tricine gel (Figure 6). The ligation reaction was tested in conditions of 5-150 mM Tris or HEPES buffers, 50-1000 mM NaCl, 10 mM Maltose, and pH 6-11 and 0-6 M Urea.

#### **Direct Ligation Reactions:**

MYBleu (1 mg/mL) was incubated in 6 M Urea or 1% SDS, pH 7.5-8.5, 50-200 mM NaCl, and 1 mM of a 30 amino acid peptide (NH<sub>2</sub>CAYKTTQANKHIVVACEGNPYVPVHFDASV-COOH (SEQ ID NO:6)). The MYBleu was incubated for 0-180 minutes at either 4°C or 100°C prior to the addition of the 30 amino acid peptide. Ligation reactions proceeded overnight at either 4°C or 25°C.

## **EXAMPLE II**

### **Labeling a target protein: Maltose Binding Protein**

5           Maltose binding protein (MBP, 42 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10           A biotinylated peptide possessing an N-terminal cysteine (CDPEK\*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the  $\epsilon$ -amino group of the lysine residue) was ligated to the freshly purified target protein as described above. Briefly, 4  $\mu$ L of biotinylated peptide (10 mM) were  
15           mixed with a 36  $\mu$ L aliquot of the freshly purified MBP sample. The mixture was incubated at 4°C overnight.

          Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product  
20           but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

**EXAMPLE III****Labeling a target protein: Bst DNA Polymerase I Large Fragment (Bst Pol 1)**

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Bst DNA Polymerase I large fragment (67 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10

A biotinylated peptide possessing an N-terminal cysteine (CDPEK\*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the  $\epsilon$ -amino group of the lysine residue) was ligated to the freshly purified target protein as described.

15

Briefly, 4  $\mu$ L of biotinylated peptide (10 mM) were mixed with a 36  $\mu$ L aliquot of the freshly purified Bst Pol 1 sample. The mixture was incubated at 4°C overnight.

20

Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

#### **EXAMPLE IV**

##### **Labeling a target protein: Paramyosin**

5           Paramyosin (29 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10           A biotinylated peptide possessing an N-terminal cysteine (CDPEK\*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the  $\epsilon$ -amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4  $\mu$ L of biotinylated peptide (10 mM) were mixed with  
15           a 36  $\mu$ L aliquot of the freshly purified paramyosin sample. The mixture was incubated at 4°C overnight.

            Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product  
20           but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

**EXAMPLE V****Labeling a target protein: *E. coli* Thioredoxin**

5           *E. coli* thioredoxin (12 kDa) was isolated as described in Example I above using the IMPACT procedure (IMPACT manual from New England Biolabs, Inc., Beverly, MA) in the presence of MESNA.

10           A biotinylated peptide possessing an N-terminal cysteine (CDPEK\*DS-COOH (SEQ ID NO:9)), in which the biotin was attached to the  $\epsilon$ -amino group of the lysine residue) was ligated to the freshly purified target protein as described. Briefly, 4  $\mu$ L of biotinylated peptide (10 mM) were mixed with  
15           a 36  $\mu$ L aliquot of the freshly purified thioredoxin sample. The mixture was incubated at 4°C overnight.

            Western blots with alkaline phosphatase linked anti-biotin antibody detected the presence of the ligated product  
20           but not the unligated target protein (Figure 7). The efficiency of the ligation is typically greater than 90% when MESNA is used for cleavage.

**EXAMPLE VI****Isolation of a cytotoxic protein:**

5           The ligation procedure of Example I was applied to the isolation of a potentially cytotoxic protein. An endonuclease from *Haemophilus parainfluenzae* (*HpaI*; Ito, et al., (1992) *Nucleic Acids Res* 20:705-709) was generated by ligating an inactive truncated form of the enzyme expressed in *E. coli*  
10           (ER2566 cells, New England Biolabs, Inc., Beverly, MA) with the missing amino acids that were synthesized chemically.

          The first 223 amino acids of *HpaI* (full length *HpaI* is 254 amino acids) were fused in frame with the modified *Mxe*  
15           GyrA intein and the CBD. The 223 amino acid *HpaI* fragment was isolated as described for purification of thioester tagged proteins. The truncated *HpaI* displayed no detectable enzymatic activity.

20           A synthetic peptide representing the 31 amino acids needed to complete *HpaI* was ligated onto the 223 amino acid truncated form of *HpaI* by the method of Example I.

**Enzymatic Assay for *HpaI*:**

The activity of the fused *HpaI* was determined by its ability to digest Lambda DNA (New England Biolabs, Beverly, MA). Serial dilutions of ligated or truncated *HpaI*, with the appropriate peptide added to 1 mM, were incubated with 1  $\mu$ g of Lambda DNA for 1 hour at 37°C in a buffer of 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, and 170  $\mu$ g/mL BSA (total volume 30  $\mu$ L). Digestion reactions were visualized on 1% agarose gels permeated with ethidium bromide. One unit of *HpaI* was defined as the amount of enzyme necessary to digest 1  $\mu$ g of Lambda DNA in one hour at 37°C.

The newly ligated *HpaI* had a specific activity of 0.5-1.5x10<sup>6</sup> units/mg which correlated well with the expected value of 1-2x10<sup>6</sup> units/mg for the full length enzyme.



**WHAT IS CLAIMED IS:**

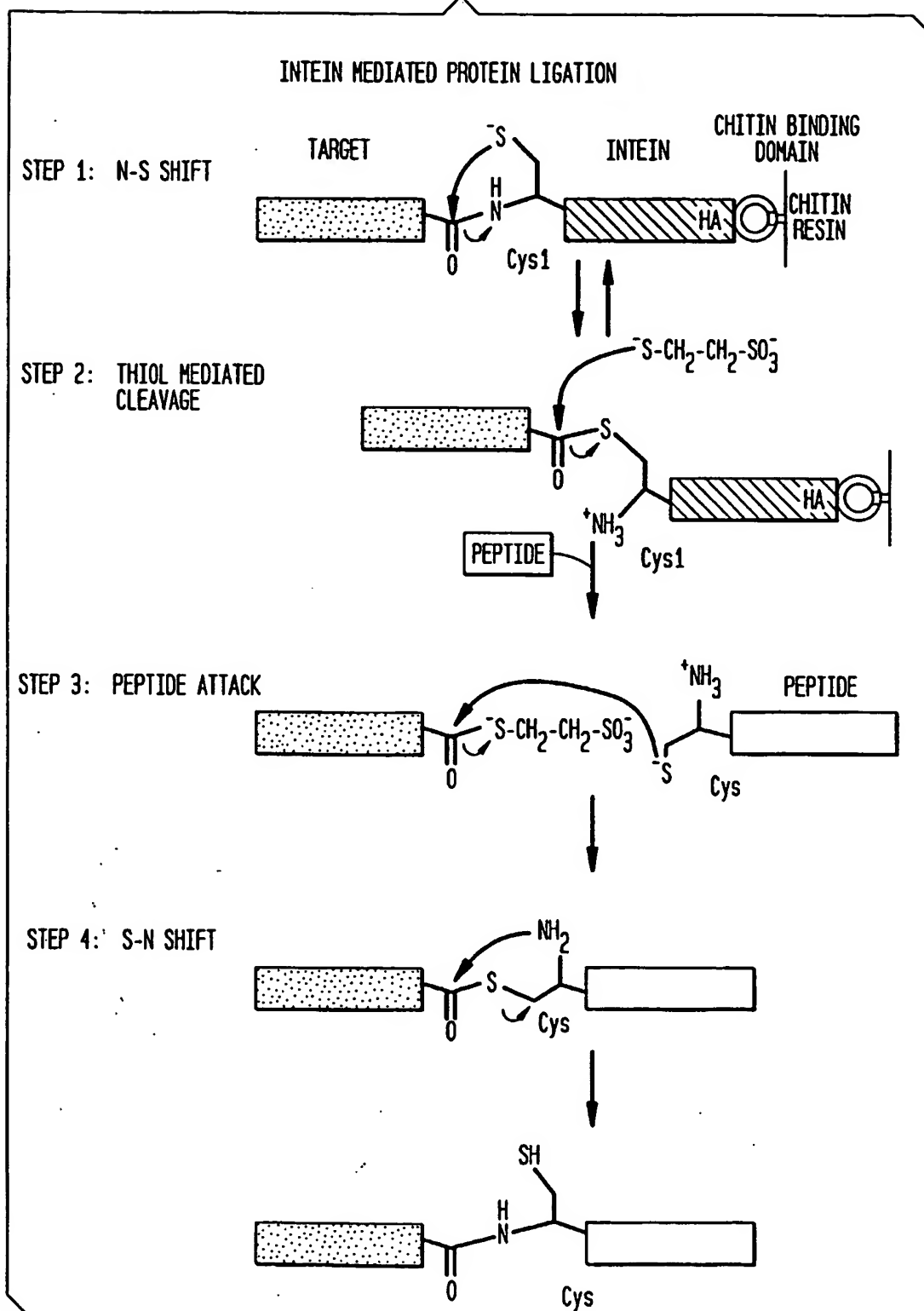
1. A method for fusing an expressed protein with a peptide,  
said method comprising the steps of:
  - 5 (a) generating at least one C-terminal thioester-tagged target protein;
  - (b) generating at least one target peptide having a specified N-terminal; and
  - (c) ligating said target peptide to said target protein.
- 10 2. The method of claim 1, wherein said target protein is generated from a first plasmid comprising an intein having N-terminal cleavage activity.
- 15 3. The method of claim 2, wherein said intein comprises an intein having a cysteine residue at the N-terminal of the intein.
- 20 4. The method of claim 3, wherein said target protein is generated by thiol reagent-induced cleavage of said intein.
- 25 5. The method of claim 4, wherein said thiol reagent is selected from the group consisting of MESNA, thiophenol, DTT,  $\beta$ -mercaptoethanol or derivatives thereof.
6. A fusion protein produced by the method of any one of claims 1-5.

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7. A cyclic protein produced by the method of claim 1.
8. A modified intein comprising a mutant Mxe GyrA intein  
5 capable of thiol reagent-induced cleavage to produce a  
thioester at the C-terminal of an adjacent target  
protein.
9. A method of generating a reactive thioester comprising  
10 contacting a thiol reagent selected from the group  
consisting essentially of MESNA, thiophenol, DTT,  $\beta$ -  
mercaptoethanol or derivatives thereof with a precursor  
comprising a target protein and intein.
- 15 10. A method for screening thiol reagents which cleave a  
target intein comprising the steps of:
  - (a) isolating a precursor comprising a protein and a  
modified intein;
  - (b) contacting a thiol reagent with the precursor of  
20 step (a);
  - (c) determining whether a splicing or cleaving event  
occurs.
- 25 11. The method of claim 10, comprising the further step of  
determining whether the spliced or cleaved product of  
step (c) can ligate to a target peptide having an N-  
terminal cytokine.

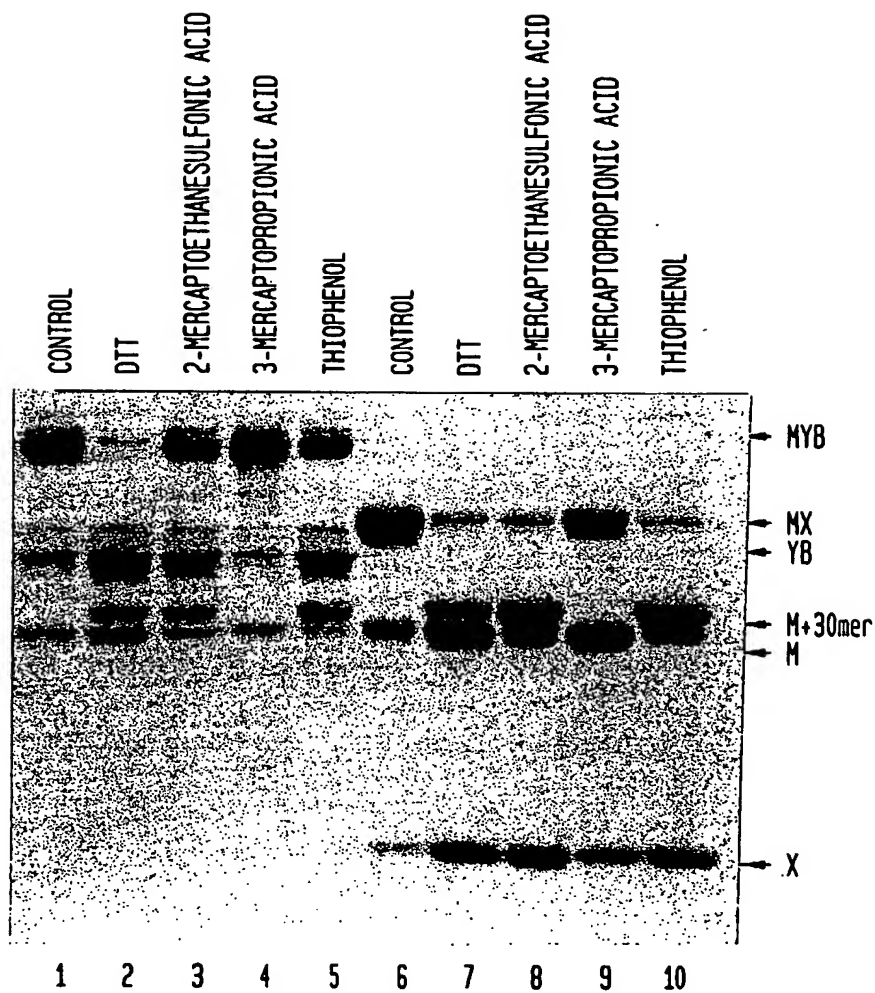
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FIG. 1



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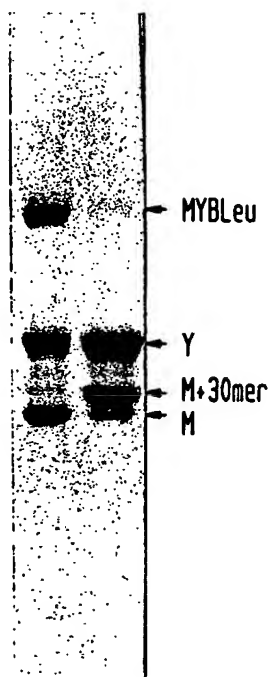
FIG. 2



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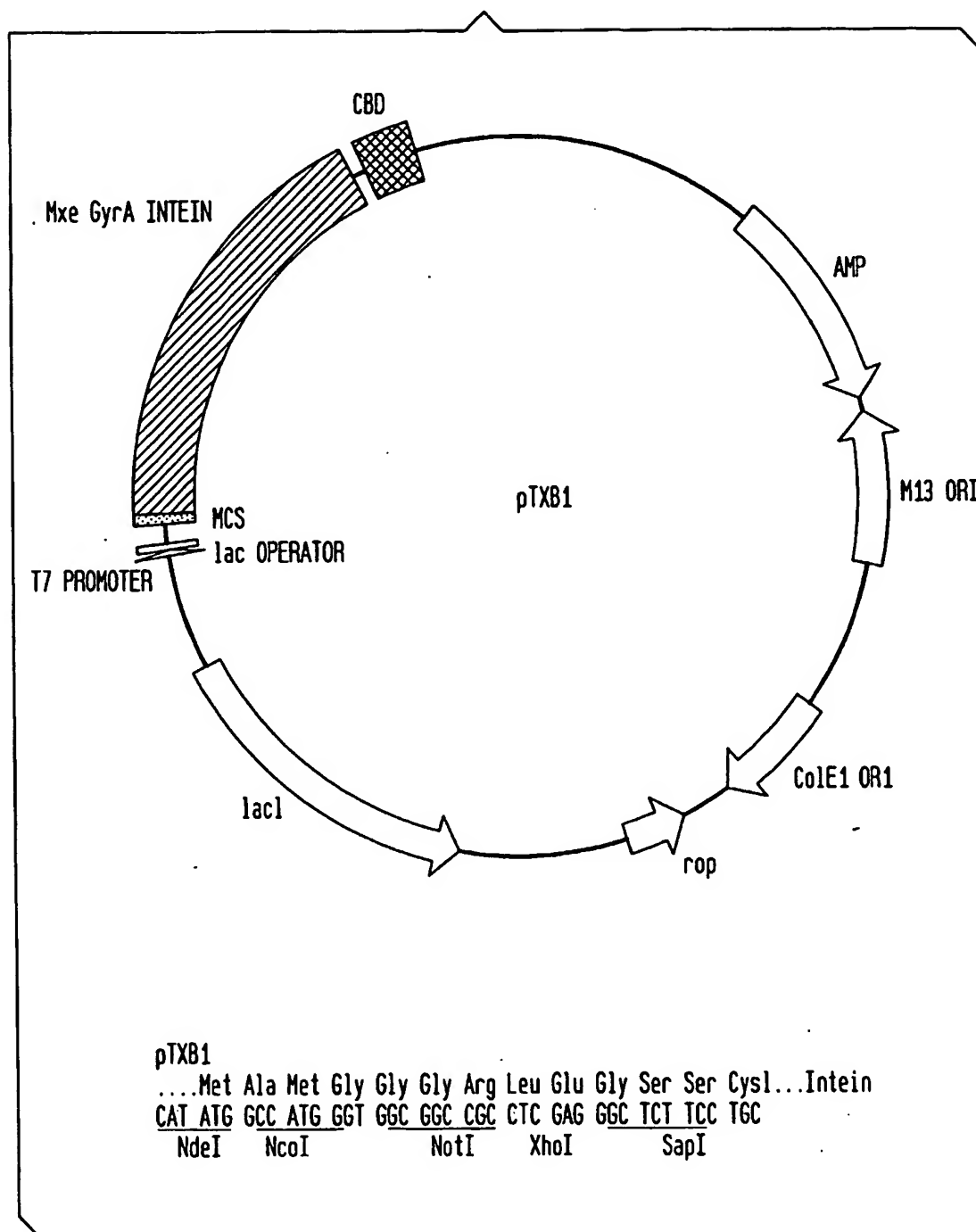
**FIG. 3**

DIRECT LIGATION REACTION



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FIG. 4



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## FIG. 5A

## DNA Sequence of pTXB1 plasmid

140- 997 beta-lactamase (Ap)  
1042-1555 M13 origin  
2254 ColE1 origin  
2626-2814 rop  
3376-4455 lacIq  
5440-5456 T7 promoter  
5440-5459 T7 universal primer (forward)  
5457 first nucleotide of the T7 transcript  
5459-5483 lac operator  
5513-5519 Shine-Dalgarno sequence (T7 gene 10)  
5525-5572 Multiple cloning site  
5573-6166 Mxe GyrA intein (N198A)  
6197-6352 Chitin-binding domain  
6375-6497 T7 transcription terminator

TXB1.seq.old Length: 6503 March 17, 1998 11:14 Type: N  
Check: 1445 ..

```
1  AACTACGTCA GGTGGCACTT TTCGGGGAAA TGTGCGCGGA ACCCCTATTT
51  GTTTATTTTT CTAAATACAT TCAAATATGT ATCCGCTCAT GAGACAATAA
101 CCCTGATAAA TGCTTCAATA ATATTGAAAA AGGAAGAGTA TGAGTATTCA
151 ACATTTCCGT GTCGCCCTTA TTCCCTTTTT TCGGCATTT TGCCTTCCTG
201 TTTTGTGCTCA CCCAGAAACG CTGGTGAAAG TAAAAGATGC TGAAGATCAG
251 TTGGGTGCAC GAGTGGGTTA CATCGAACTG GATCTCAACA GCGGTAAGAT
301 CCTTGAGAGT TTTCGCCCCG AAGAACGTTT TCCAATGATG AGCACTTTTA
351 AAGTCTGCT ATGTGGCGCG GTATTATCCC GTGTGACGC CGGGCAAGAG
401 CAACTCGGTC GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC
451 ACCAGTCACA GAAAAGCATC TTACGGATGG CATGACAGTA AGAGAATTAT
501 GCAGTGCTGC CATAACCATG AGTGATAACA CTGCGGCCAA CTTACTTCTG
551 ACAACGATCG GAGGACCGAA GGAGCTAACC GCTTTTTTGC ACAACATGGG
601 GGATCATGTA ACTCGCCTTG ATCGTTGGGA ACCGGAGCTG AATGAAGCCA
651 TACCAAACGA CGAGCGTGAC ACCACGATGC CTGTAGCAAT GGCAACAACG
701 TTGCGCAAAC TATTAAC TGG CGAACTACTT ACTCTAGCTT CCCGGCAACA
751 ATTAATAGAC TGGATGGAGG CGGATAAAGT TGCAGGACCA CTTCTGCGCT
801 CGGCCCTTCC GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG
851 CGTGGGTCTC GCGGTATCAT TGCAGCACTG GGGCCAGATG GTAAGCCCTC
```

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**FIG. 5B**

901 CCGTATCGTA GTTATCTACA CGACGGGGAG TCAGGCAACT ATGGATGAAC  
951 GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA GCATTGGTAA  
1001 CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TACCCCGGTT  
1051 GATAATCAGA AAAGCCCCAA AAACAGGAAG ATTGTATAAG CAAATATTTA  
1101 AATTGTAAAC GTTAATATTT TGTAAAATT CGCGTTAAAT TTTTGTAA  
1151 TCAGCTCATT TTTTAACCA TAGGCCGAAA TCGGCAAAAT CCCTTATAAA  
1201 TCAAAAGAAT AGCCCGAGAT AGGGTTGAGT GTTGTTCAG TTTGGAACAA  
1251 GAGTCCACTA TTAAAGAACG TGGACTCCAA CGTCAAAGGG CGAAAAACCG  
1301 TCTATCAGGG CGATGGCCCA CTACGTGAAC CATCACCCAA ATCAAGTTTT  
1351 TTGGGGTCGA GGTGCCGTAA AGCACTAAAT CGGAACCCTA AAGGGAGCCC  
1401 CCGATTTAGA GCTTGACGGG GAAAGCCGGC GAACGTGGCG AGAAAGGAAG  
1451 GGAAGAAAGC GAAAGGAGCG GCGCTAGGG CGCTGGCAAG TGTAGCGGTC  
1501 ACGCTGCGCG TAACCACCAC ACCCGCCGCG CTTAATGCGC CGCTACAGGG  
1551 CGCGTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA  
1601 ATCCCTTAAC GTGAGTTTTT GTTCCACTGA GCGTCAGACC CCGTAGAAAA  
1651 GATCAAAGGA TCTTCTGAG ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT  
1701 TGCAAACAAA AAAACCACCG CTACCAGCGG TGGTTTGTTT GCCGGATCAA  
1751 GAGCTACCAA CTCTTTTTTC GAAGGTAAC GGCTTCAGCA GAGCGCAGAT  
1801 ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA  
1851 ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG  
1901 GCTGCTGCCA GTGGCGATAA GTCGTGCTT ACCGGGTTGG ACTCAAGACG  
1951 ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTGCTGCA  
2001 CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG  
2051 CGTGAGCTAT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA AGCGGACAG  
2101 GTATCCGGTA AGCGGCAGGG TCGGAACAGG AGAGCGCACG AGGGAGCTTC  
2151 CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC  
2201 TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG  
2251 GAAAAACGCC AGCAACCGCG CCTTTTACG GTTCCTGGCC TTTTGCTGGC  
2301 CTTTGTCTCA CATGTTCTTT CTGCGTTAT CCCCTGATC TGTGGATAAC



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## FIG. 5C

2351 CGTATTACCG CCTTTGAGTG AGCTGATACC GCTCGCCGCA GCCGAACGAC  
2401 CGAGCGCAGC GAGTCAGTGA GCGAGGAAGC TATGGTGCAC TCTCAGTACA  
2451 ATCTGCTCTG ATGCCGCATA GTTAAGCCAG TATACACTCC GCTATCGCTA  
2501 CGTGACTGGG TCATGGCTGC GCCCCGACAC CCGCCAACAC CCGCTGACGC  
2551 GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA CAAGCTGTGA  
2601 CCGTCTCCGG GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCAGAA  
2651 CGCGCGAGGC AGCTGCGGTA AAGCTCATCA GCGTGGTCGT GCAGCGATTC  
2701 ACAGATGTCT GCCTGTTTCT CCGCGTCCAG CTCGTTGAGT TTCTCCAGAA  
2751 GCGTTAATGT CTGGCTTCTG ATAAAGCGGG CCATGTTAAG GCGGTTTTTT  
2801 TCCTGTTTTG TCACTTGATG CCTCCGTGTA AGGGGGAATT TCTGTTTCATG  
2851 GGGGTAATGA TACCGATGAA ACGAGAGAGG ATGCTCACGA TACGGGTTAC  
2901 TGATGATGAA CATGCCCGGT TACTGGAACG TTGTGAGGGT AAACAACCTGG  
2951 CCGTATGGAT GCGGCGGGAC CAGAGAAAAA TCACTCAGGG TCAATGCCag  
3001 ccgaACGCCA GCAAGACGTA GCCCAGCGCG TCGGCCGCCA TGCCGGCGAT  
3051 AATGGCCTGC TTCTCGCCGA AACGTTTGGT GCGGGGACCA GTGACGAAGG  
3101 CTTGAGCGAG GCGGTGCAAG ATTCCGAATA CCGCAAGCGA CAGGCCGATC  
3151 ATCGTCGCGC TCCAGCGAAA GCGGTCCTCG CCGAAAATGA CCCAGAGCGC  
3201 TGCCGGCACC TGTCTTACGA GTTGCATGAT AAAGAAGACA GTCATTAAGTG  
3251 CGGCGACGAT AGTCATGCCC CGCGCCACC GGAAGGAGCT GACTGGGTTG  
3301 AAGGCTCTCA AGGGCATCGG TCGAGATCCC GGTGCCTAAT GAGTGAGCTA  
3351 ACTTACATTA ATTGCGTTGC GCTCACTGCC CGCTTTCCAG TCGGGAAACC  
3401 TGTCGTGCCA GCTGCATTAA TGAATCGGCC AACGCGCGGG GAGAGGCGGT  
3451 TTGCGTATTG GCGGCCAGGG TGGTTTTTCT TTTCACCAGT GAGACGGGCA  
3501 ACAGCTGATT GCCCTTACC GCCTGGCCCT GAGAGAGTTG CAGCAAGCGG  
3551 TCCACGCTGG TTTGCCCCAG CAGGCGAAAA TCCTGTTTGA TGGTGGTTAA  
3601 CGGCGGGATA TAACATGAGC TGTCTTCGGT ATCGTCGTAT CCCACTACCG  
3651 AGATATCCGC ACCAACGCGC AGCCCGGACT CGGTAATGGC GCGCATTGCG  
3701 CCCAGCGCCA TCTGATCGTT GGCAACCAGC ATCGCAGTGG GAACGATGCC  
3751 CTCATTACAG ATTGTCATGG TTTGTTGAAA ACCGGACATG GCACTCCAGT

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## FIG. 5D

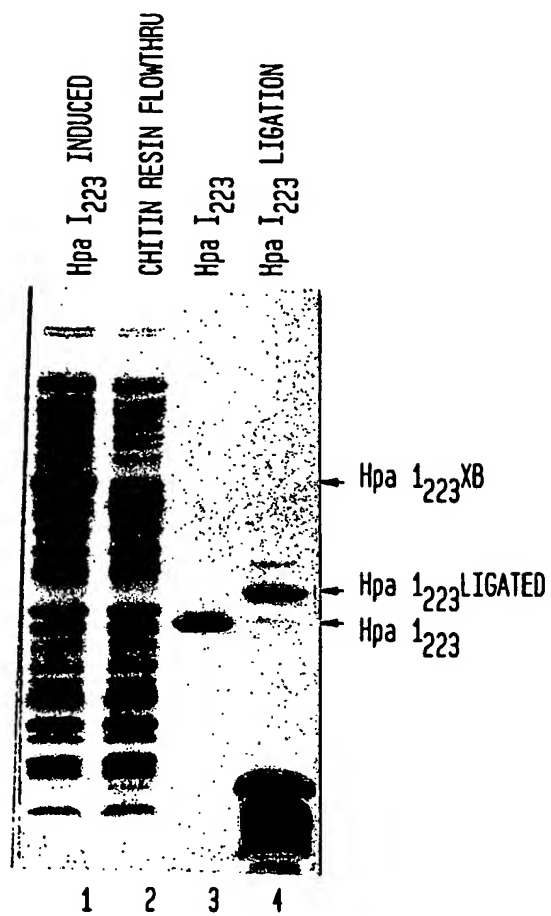
3801 CGCCTTCCCG TTCCGCTATC GGCTGAATTT GATTGCGAGT GAGATATTTA  
3851 TGCCAGCCAG CCAGACGCAG ACGCGCCGAG ACAGAACTTA ATGGGCCCCG  
3901 TAACAGCGCG ATTTGCTGGT GACCCAATGC GACCAGATGC TCCACGCCCA  
3951 GTCGCGTACC GTCTTCATGG GAGAAAATAA TACTGTTGAT GGGTGTCTGG  
4001 TCAGAGACAT CAAGAAATAA CGCCGGAACA TTAGTGCAGG CAGCTTCCAC  
4051 AGCAATGGCA TCCTGGTCAT CCAGCGGATA GTTAATGATC AGCCCACTGA  
4101 CGCGTTGCGC GAGAAGATTG TGCACCGCCG CTTTACAGGC TTCGACGCCG  
4151 CTTGTTCTTA CCATCGACAC CACCACGCTG GCACCCAGTT GATCGGCGCG  
4201 AGATTTAATC GCCGCGACAA TTTGCGACGG CGCGTGCAGG GCCAGACTGG  
4251 AGGTGGCAAC GCCAATCAGC AACGACTGTT TGCCCGCCAG TTGTTGTGCC  
4301 ACGCGGTTGG GAATGTAATT CAGCTCCGCC ATCGCCGCTT CCACTTTTTC  
4351 CCGCGTTTTT GCAGAAACGT GGCTGGCCTG GTTACCACG CGGGAACGG  
4401 TCTGATAAGA GACACCGGCA TACTCTGCGA CATCGTATAA CGTTACTGGT  
4451 TTCACATTCA CCACCCTGAA TTGACTCTCT TCCGGGCGCT ATCATGCCAT  
4501 ACCGCGAAAG GTTTTGCGCC ATTCGATGGT GTCCCGGATC TCGACGCTCT  
4551 CCCTTATGCG ACTCCTGCAT TAGGAAGCAG CCCAGTAGTA GGTGAGGCC  
4601 GTTGAGCACC GCCGCCGCAA GGAATGGTGC ATGCCGCCCT TTCGTCTTCA  
4651 AGAATTAATT CCCAATTCCA GGCATCAAAT AAAACGAAAG GCTCAGTCGA  
4701 AAGACTGGGC CTTTCGTTTT ATCTGTTGTT TGTCGGTGAA CGCTCTCCTG  
4751 AGTAGGACAA ATCCGCCGGG AGCGGATTG AACGTTGCGA AGCAACGGCC  
4801 CGGAGGGTGG CGGGCAGGAC GCCCGCCATA AACTGCCAGG AATTAATTCC  
4851 AGGCATCAAA TAAAACGAAA GGCTCAGTCG AAAGACTGGG CCTTTCGTTT  
4901 TATCTGTTGT TTGTCGGTGA ACGCTCTCCT GAGTAGGACA AATCCGCCGG  
4951 GAGCGGATTI GAACGTTGCG AAGCAACGGC CCGGAGGGTG GCGGGCAGGA  
5001 CGCCCGCCAT AACTGCCAG GAATTAATTC CAGGCATCAA ATAAAACGAA  
5051 AGGCTCAGTC GAAAGACTGG GCCTTTCGTT TTATCTGTTG TTTGTCGGTG  
5101 AACGCTCTCC TGAGTAGGAC AAATCCGCCG GGAGCGGATT TGAACGTTGC  
5151 GAAGCAACGG CCCGGAGGGT GCGGGCAGG ACGCCGCCA TAAACTGCCA  
5201 GGAATTAATT CCAGGCATCA AATAAACGA AAGGCTCAGT CGAAAGACTG

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## FIG. 5E

5251 GGCCTTTCGT TTTATCTGTT GTTTGTCGGT GAACGCTCTC CTGAGTAGGA  
5301 CAAATCCGCC GGGAGCGGAT TTGAACGTTG CGAAGCAACG GCCCGGAGGG  
5351 TGGCGGGCAG GACGCCCGCC ATAAACTGCC AGGAATTGGG GATCGGAATT  
5401 AATTCCCGGT TTAAACCGGG GATCTCGATC CCGCGAAATT AATACGACTC  
5451 ACTATAGGGG AATTGTGAGC GGATAACAAT TCCCCTCTAG AAATAATTTT  
5501 GTTTAACTTT AAGAAGGAGA TATAcatatg gctagctcgc gagtgcagcg  
5551 cggccgcctc gagggctctt ccTGCATCAC GGGAGATGCA CTAGTTGCCC  
5601 TACCCGAGGG CGAGTCGGTA CGCATCGCCG ACATCGTGCC GGGTGCCCGG  
5651 CCCAACAGTG ACAACGCCAT CGACCTGAAA GTCCTTGACC GGCATGGCAA  
5701 TCCCGTGCTC GCCGACCGGC TGTTCCTACTC CGGCGAGCAT CCGGTGTACA  
5751 CGGTGCGTAC GGTGGAAGGT CTGCGTGTGA CGGGCACCCG GAACCACCCG  
5801 TTGTTGTGTT TGGTCGACGT CGCCGGGGTG CCGACCCTGC TGTGGAAGCT  
5851 GATCGACGAA ATCAAGCCGG GCGATTACGC GGTGATTCAA CGCAGCGCAT  
5901 TCAGCGTCGA CTGTGCAGGT TTTGCCCGCG GAAAACCCGA ATTTGCGCCC  
5951 ACAACCTACA CAGTCGGCGT CCCTGGACTG GTGCGTTTCT TGGAAGCACA  
6001 CCACCGAGAC CCGACGCCC AAGCTATCGC CGACGAGCTG ACCGACGGGC  
6051 GGTTCCTACTA CGCGAAAGTC GCCAGTGTCA CCGACGCCGG CGTGCAGCCG  
6101 GTGTATAGCC TTCGTGTCGA CACGGCAGAC CACGCGTTTA TCACGAACGG  
6151 GTTCGTCAGC CACGCTACTG GCCTCACC GG TCTGAACTCA GGCCTCACGA  
6201 CAAATCCTGG TGTATCCGCT TGGCAGGTCA ACACAGCTTA TACTGCGGGA  
6251 CAATTGGTCA CATATAACGG CAAGACGTAT AAATGTTTGC AGCCCCACAC  
6301 CTCCTTGGCA GGATGGGAAC CATCCAACGT TCCTGCCTTG TGGCAGCTTC  
6351 AATGactgca ggaaggGGAT CCGGCTGCTA ACAAAGCCCG AAAGGAAGCT  
6401 GAGTTGGCTG CTGCCACCGC TGAGCAATAA CTAGCATAAC CCCTTGGGGC  
6451 CTCTAAACGG GTCTTGAGGG GTTTTTTGCT GAAAGGAGGA ACTATATCCG  
6501 GAT

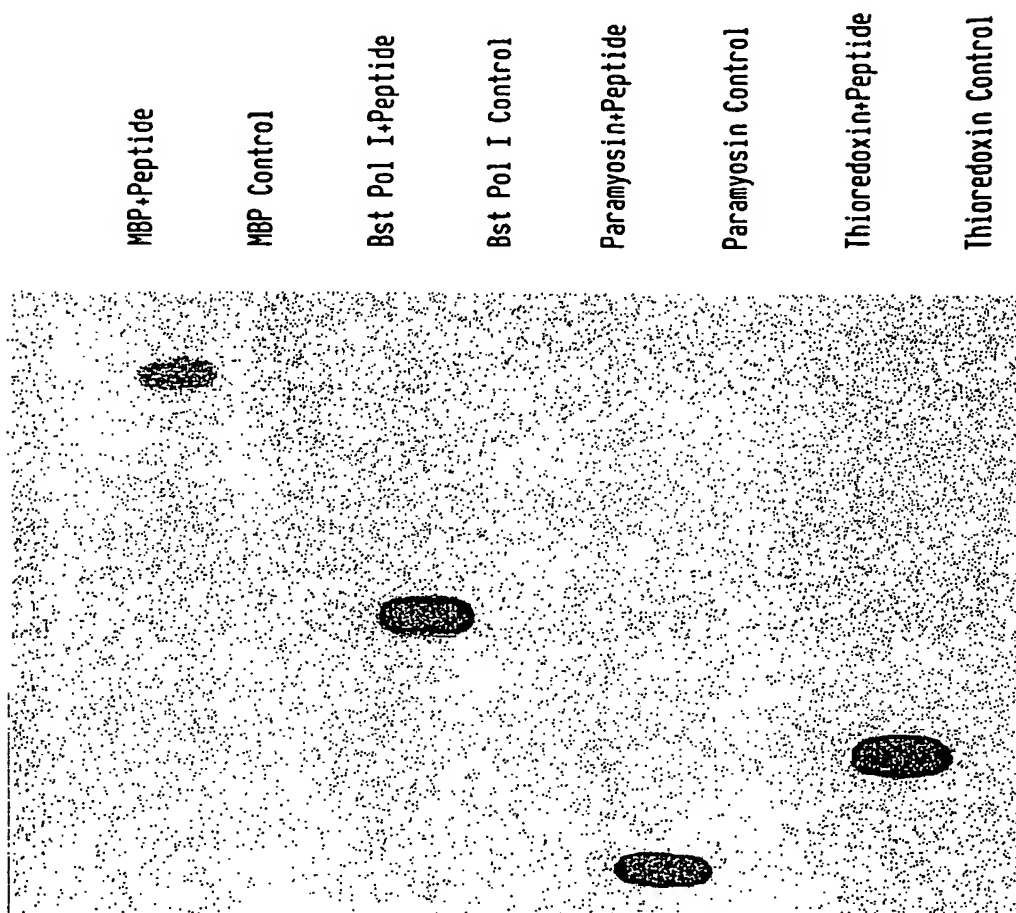
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**FIG. 6****Hpa I LIGATION**

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**FIG. 7**

WESTERN BLOTS OF PROTEINS LIGATED TO A BIOTINYLATED PEPTIDE



## INTERNATIONAL SEARCH REPORT

International application No.

BCT/US99/22776

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC(7) : C07K 19/00; C12N 9/10, 15/62; C12P 21/02, 21/04 US CL : 435/68.1, 69.7; 513/2; 530/402, 408; 536/23.4 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/68.1, 69.7; 513/2; 530/402, 408; 536/23.4		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,834,247 A (COMB et al) 10 November 1998, cols. 18-19, 40-45, 47-49, 75-77, and 87-89.	1-6, 9
X	CHONG, S. et al. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene. 19 June 1997, Vol. 192, No. 2, pages 271-281, especially Figure 1B at page 273 and pages 274-277.	9
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Y		1-6, 10
X,P	EVANS, T.C. et al. Semisynthesis of cytotoxic proteins using a modified protein splicing element. Protein Science. 05 November 1998, Vol. 7, No. 11, pages 2256-2264, especially Figure 1B at page 2258 and pages 2257 and 2259-2261.	1-6, 9
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer WILLIAM W. MOORE JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No. (703) 308-0196	

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International application No.

PCT/US99/22776

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	MUIR, T.W. et al. Expressed protein ligation: A general method for protein engineering. Proceedings of the National Academy of Sciences, U.S.A. June 1998, Vol. 95, pages 6705-6710, see entire publication.	1-6, 9 ----- 7, 8, 10
X,P	EVANS, T.C. et al. The Cyclization and Polymerization of Bacterially Expressed Proteins Using Modified Self-splicing Inteins. The Journal of Biological Chemistry. June 1999, Vol 274. No. 26, pages 18359-18363, especially pages 118360-118362.	1-7, 9
Y	TELENTI, A. et al. The Mycobacterium xenopi GyrA Protein Splicing Element: Characterization of a Minimal Intein. Journal of Bacteriology. 14 October 1997, Vol. 179, No. 20, pages 6378-6382, especially pages 6379-6381.	8

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